5711

MYCOTOXINS

Rapid Liquid Chromatographic Determination of Aflatoxins M_1 and M_2 in Artificially Contaminated Fluid Milks: Collaborative Study

ROBERT D. STUBBLEFIELD and WILLIAM F. KWOLEK[1]
U.S. Department of Agriculture, Agricultural Research Service, Northern Regional Research Center, Peoria, IL 61604

Collaborators: R. Beaver; M. Billotte; J. Ferguson-Foos; J. I. Greer; K. D. Johnson; R. Lyon; E. H. Marth; J. I. Pitt; M. E. Rhodes; J. L. Richard; B. Roberts; G. Rusul; Y. Saito; E. A. Sizoo; G. Sutton; N. F. Tobin; M. Trucksess; H. P. van Egmond; J. D. Warren; N. Webb; D. Wilson; A. E. Yousef

An international collaborative study involving 14 collaborators from 5 different countries was conducted to test a rapid liquid chromatographic (LC) method for detecting aflatoxins M_1 and M_2 in fluid milk. Each collaborator prepared artificially contaminated milk samples (0.078–1.31 ng M_1/mL and 0.030–0.13 ng M_2/mL) by adding solutions containing various concentrations of aflatoxins M_1 and M_2 to fresh milk. Recoveries ranged from 85.2 to 102.5% (av. 93.7%) for aflatoxin M_1 and from 99.5 to 126.7% (av. 109.8%) for aflatoxin M_2. Coefficients of variation averaged 21.4% (M_1) and 35.9% (M_2). An analysis of variance was calculated from combined data to determine variance components. The within-laboratory variations (S_o) (repeatability) were 27.9% (M_1) and 23.9% (M_2), and the among-laboratory variations (S_x) (reproducibility) were 44.5% (M_1) and 64.7% (M_2). No visual differences were determined between normal or reverse phase LC for contaminated samples; however, there were an insufficient number of collaborators using normal phase to give meaningful separate statistical data. For 26 observations of uncontaminated milk, 3 false M_1 positives were reported for normal phase LC determinations and 2 false M_1 positives were reported for reverse phase LC determinations. Three normal phase and 11 reverse phase false M_2 positives were reported for 104 observations in uncontaminated milk. The reverse phase LC method for determination of aflatoxins M_1 and M_2 in fluid milk has been adopted official first action.

In 1979, an international collaborative study (1) was conducted to test the Stubblefield method (2) for determining aflatoxin M_1 in dairy products and the van Egmond et al. method (3) for thin layer chromatographic (TLC) confirmation of M_1 identity. Subsequently, both methods were adopted by AOAC and the Commission of Food Contaminants of the International Union of Pure and Applied Chemistry (IUPAC) (4). The analytical method (2) uses TLC for the quantitation step. Several methods that incorporate liquid chromatography (LC) for determining M_1 in milk have been published (5–11). Some of these methods utilize disposable silica gel and/or bonded silica gel (C_{18}) cartridges to reduce analysis time. Minimum detection limits for the LC methods range from 0.01 to 0.10 ppb aflatoxin M_1 and can be adjusted readily within this range as required by the guidelines or tolerances of state, federal, or international agencies.

The Associate Referee evaluated each method to determine which one(s) provided: (*1*) interference-free extracts; and (*2*) satisfactory recoveries (>80%) of added M_1. From the data and comments of other scientists (personal communications), the Foos and Warren method (11) was selected for international collaborative study. This method, as published, was developed for normal phase LC. It is desirable that the official AOAC method be applicable to reverse phase LC, also. The published procedures for reverse phase LC of aflatoxin M_1 (6–9) were investigated, and a modification of the Beebe and Takahashi method (6) was selected. This method measures the fluorescent derivative formed by treating M_1 with trifluoroacetic acid (TFA). In aqueous mobile phase solvents, the derivative is more fluorescent than M_1 itself.

In 1983, an international collaborative study was initiated; however, incomplete reaction of standard aflatoxin M_1 and TFA occurred, and the study was halted. These problems were essentially eliminated by forming the standard derivative in a silylated glass vial to prevent irreversible adsorption of the M_1–M_2 standards to the glass walls (future publication). Adsorption causes incomplete reaction between M_1 and TFA. Further research by the Associate Referee has shown that an increase in the reaction temperature (40°C) is necessary to achieve complete derivatization.

All attempts to find a suitable preservative for naturally contaminated fluid milk were unsuccessful. Chemicals were found which preserved the milk; however, the M_1 in the milk was degraded. Consequently, collaborators were asked to furnish uncontaminated milk and to prepare artificially contaminated milk samples with sealed acetonitrile solutions of aflatoxins M_1 and M_2. The report of the data submitted by 14 collaborators from 5 different countries is presented here.

Collaborative Study

Aflatoxin M_1–M_2 Standard Solutions

Crystalline aflatoxins M_1 and M_2 were used to prepare stock solutions of each aflatoxin (266.07 μg M_1/mL and 172.02 μg M_2/mL, in acetonitrile). Aflatoxin concentrations in the stock solutions were determined according to **26.004–26.011** (12), using extinction coefficients of 19 850 and 21 400 for M_1 and M_2, respectively, in acetonitrile. Purity criteria for crystalline M_1 and M_2 are given by Stubblefield et al. (13, 14). The stock solutions were used to prepare a standard solution for LC, containing 0.50 μg M_1 and 0.10 μg M_2/mL in acetonitrile–benzene (1 + 9).

Preparation of Samples

All samples in the study were artifically contaminated fluid milks. Seven aflatoxin M_1–M_2 spiking solutions (in acetonitrile) were prepared from aliquots of M_1 and M_2 stock or diluted stock solutions in separate 100 mL volumetric flasks as follows: Samples 1/8, and practice, 29.1 μL stock M_1 with 37.1 μL 10-fold dilution M_2; samples 2/9, 58.2 μL 10-fold dilution stock M_1; samples 3/10, 29.2 μL 10-fold dilution stock

Submitted for publication October 1, 1985.
This report of the Associate Referee, R. D. Stubblefield, was presented at the AOAC 10th Annual Spring Training Workshop, April 9–11, 1985, at Dallas, TX.
The recommendation of the Associate Referee was approved by the General Referee and the Committee on Foods I and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1986) **69**, March issue.
The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.
[1]Retired.

Table 1. Collaborative results for determination of aflatoxins M_1 and M_2 (ng/mL) in artificially contaminated fluid milk (blind duplicate pairs)[a]

		Sample 1		Sample 8		Sample 2		Sample 9		Sample 3		Sample 10	
Coll.	Method[b]	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2
1[c]	R	0.32	0.01	0.20	0.03	0.05	0	0.05	0	0.05	0	0.03	0
4	R	0.129[d]	0.010	0.287	0.023	0.112	0	0.132	0	0.073	0	0.034	0
6	R	0.85	0.07	1.07	0.10	0.20	0.02	0.16	0	0.06	0	0.06	0
8	N	0.80	0.10	0.81	0.09	0.12	0.017	0.10	0	0.06	0	0.06	0
18	R	0.72	0.05	0.68	0.06	0.22	0.01	0.10	0	0.07	0	0.08	0
19	R	0.76	0.16	0.18	0	0.074	0.18	0.19	0	0.048	0.17	0.081	0.082
20	N	0.85	0.08	0.83	0.08	0.17	0	0.21	tr[e]	0.07	0	0.11	0
21	N	0.7	0	0.7	0	0.3	0	0.3	0	0.2[d]	0	0.2[d]	0
22	R	0.81	0.1	0.73	0.08	0.20	0	0.20	0	0.11	0	0.12	0
23	R	0.835	0.084	0.925	0.086	0.165	0	0.085	0	0.095	0.02	0.076	0.05
25	R	0.57	0.077	0.71	0.055	0.15	0	0.13	0	0.063	0	0.046	0
26	R	0.77	0.06	0.73	0.08	0.18	0	0.18	0	0.09	0	0.10	0
27	R	0.49	0.03	0.30	0.05	0.07	0	0.10	0	0.02	0	0.02	0
33	R	0.75	0.09	0.70	0.08	0.25	0	0.15	0	0.20[d]	0	0.20[d]	0
		Sample 5		Sample 12		Sample 6		Sample 13		Sample 7		Sample 14	
Coll.	Method[b]	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2
1[c]	R	0.08	<0.01	0	0	0.30	0.05	0.26	0.03	0.06	0	0.02	0
4	R	0.360	0.026	0.183	0.015	0.815	0.075	0.512	0.055	0.069	0	0.044	0
6	R	0.54	0.05	0.70	0.04	1.59	0.145	1.71	0.18	0.17	0.01	0.14	0
8	N	0.44	tr[e]	0.51	0.05	1.16	0.17	1.25	0.17	0.12	0	0.11	0.017
18	R	0.39	0.04	0.53	0.03	1.25	0.1	1.61	0.14	0.12	0	0.07	0
19	R	0.050[d]	0.13	0.30	0.11	0.93	0.15	1.79	2.1[d]	0.11	0.11	0.10	0
20	N	0.32	0	0.54	0.03	1.40	0.14	1.34	0.18	0.14	0	0.12	0
21	N	lost[f]	lost[f]	0.5	0	0.8	tr[e]	1.0	tr[e]	0.02	0	0.2	0
22	R	0.46	0	0.52	0	1.28	0.13	1.16	0.14	0.14	0	0.14	0
23	R	0.415	0.03	0.535	0.04	2.035	0.189	1.365	0.16	0.085	0.01	0.175	0.02
25	R	0.25	0.043	0.48	0.037	1.38	0.12	1.3	0.11	0.10	0	0.11	0
26	R	0.40	0.06	0.44	0.04	1.35	0.13	1.24	0.12	0.12	0	0.12	0
27	R	0.41	0.03	0.44	0.04	1.18	0.11	1.30	0.13	0.11	0	0.11	0
33	R	0.50	0.08	1.00[d]	0.13	1.75	0.25	1.60	0.25	0.21	0	0.24	0

[a]Duplicate pairs: 1/8; 2/9; 3/10; 5/12; 6/13; 7/14.
[b]Determined by LC method of Foos and Warren (11). N = normal phase; R = reverse phase.
[c]Values omitted from calculations after applying Youden's ranking test (16).
[d]Values omitted from calculations as outlined by Dixon's test (15).
[e]Collaborator reported trace. Trace was taken as 0.005 ng M_2/mL for statistical purposes.
[f]Sample lost.

M_1; samples 4/11, no aflatoxin; samples 5/12, 17.5 μL stock M_1 with 18.8 μL 10-fold dilution stock M_2; samples 6/13, 49.4 μL stock M_1 with 75.2 μL 10-fold dilution stock M_2; and samples 7/14, 43.6 μL 10-fold dilution stock M_1. All sample solutions and LC standard solutions were dispensed into 2 mL glass ampules (1.5 mL each), and the glass ampules were sealed. The final aflatoxin concentrations in milk were 0.078–1.31 ng M_1/mL and 0.030–0.13 ng M_2/mL.

Description of Studies

Each of 14 collaborators received the following items: 1 ampule of aflatoxin M_1–M_2 standard solution (0.50 ng M_1 and 0.10 ng M_2/mL, in acetonitrile–benzene, 1 + 9); 1 ampule of TFA; 1 ampule of dichlorodimethylsilane (DDS); 1 ampule of practice milk-contaminating solution (0.77 ng M_1, and 0.06 ng M_2/mL milk); 14 coded ampules containing milk-contaminating solutions (in acetonitrile); 5 polypropylene Econo-Columns with 35 μm support disk (Bio-Rad); 17 Sep-Pak C_{18} cartridges (Waters Associates); 20 g E. Merck silica gel 60, particle size 0.040–0.063 mm (No. 9385) containing 1% water; 5 disposable syringe tips for rubber stopper vacuum filtration; two 30 mL polypropylene syringes; two 10 mL polypropylene syringes; and a copy of study instructions, method description, and report sheet. Collaborators were required to furnish domestic aflatoxin-free milk to prepare the artificially contaminated milk samples. For each sample, collaborators were to quantitatively pipet 1.0 mL acetonitrile solution from a sample ampule into a 100 mL graduate containing about 50 mL milk. They were to add additional milk to the 100 mL mark, pour the spiked milk into a beaker to mix, and use 20 mL of the solution for analysis as described in the method. This procedure diluted the acetonitrile solution to prevent early elution of M_1 and/or M_2 from the C_{18} Sep-Pak cartridges during the extraction step.

Sample solutions were prepared which would test the method at levels that have been reported in commercial milks. Each collaborator's samples were assigned a different set of computer-selected random numbers from 1 to 14. Each sample had a duplicate in the set (7 sets of blind duplicates).

Aflatoxins M_1 and M_2 in Fluid Milk

Liquid Chromatographic Method

First Action

26.B02 *Principle*

Aflatoxins M_1 and M_2 are extd from milk on C18 cartridge, eluted with ether onto silica column, eluted with CH_2Cl_2–alcohol, and derivatized with trifluoroacetic acid. Liq. chromatgc peaks are detected fluorometrically and compared with std-TFA derivatives.

26.B03 *Reagents*

(a) *Solvents.*—Distd in glass CH_3CN, CH_2Cl_2, and isopropyl alcohol; reagent grade alcohol, ether (0.01% EtOH preservative), hexane, MeOH, trifluoroacetic acid, and H_2O (deionized, filtered thru 0.45 μm filter).

(b) *Water–acetonitrile wash soln.*—95 + 5.

(c) *Methylene chloride–alcohol elution soln.*—95 + 5.

(d) *Mobile phase.*—Prep. H_2O–isopropyl alcohol–CH_3CN (80 + 12 + 8). Degas in ultrasonic bath, or equiv. Alternative solv. proportions may be used to give optimum resolution (i.e., 84 + 11 + 5).

Table 2. Statistical evaluation of results for LC determination of aflatoxins M_1 and M_2 in artificially contaminated fluid milk[a]

Statistic	Sample 1/8	Sample 2/9	Sample 3/10	Sample 5/12	Sample 6/13	Sample 7/14
			Aflatoxin M_1			
Mean, ng/mL	0.656 (0.603)[b]	0.155 (0.143)	0.072 (0.067)	0.412 (0.415)	1.235 (1.300)	0.123 (0.116)
Std dev.	0.131	0.043	0.016	0.079	0.243	0.024
Coeff. of var., %	19.9	27.7	22.2	19.2	19.7	19.5
Theoretical, ng/mL	0.77	0.155	0.078	0.47	1.31	0.12
Recovery, %	85.2	100.0	92.3	87.7	94.3	102.5
N[c]	25 (20)	26 (20)	22 (18)	23 (18)	26 (20)	26 (20)
			Aflatoxin M_2			
Mean, ng/mL	0.062 (0.065)			0.038 (0.050)	0.128 (0.134)	
Std dev.	0.033			0.016	0.016	
Coeff. of var., %	53.2			42.1	12.5	
Theoretical, ng/mL	0.06			0.03	0.13	
Recovery, %	103.3			126.7	99.5	
N[c]	26 (20)			25 (20)	25 (19)	

[a]Calculated from values in Table 1.
[b]Values calculated after omitting data obtained using normal phase LC (Collaborators 8, 20, 21).
[c]N = number of values.

(e) *Aflatoxin std solns.*—Aflatoxin M_1 (Eureka Laboratories, Sacramento, CA 95816) and aflatoxin M_2 (Sigma Chemical Co.). Prep. stock solns (ca 200 μg M_1/mL and 100 μg M_2/mL) in CH_3CN and det. concns according to **26.004–26.011,** using extinction coefficients of 19 850 and 21 400 for M_1 and M_2, resp., in CH_3CN. Make working std soln contg 0.50 μg M_1 and 0.10 μg M_2/mL in CH_3CN–benzene (1 + 9) for use in prepg M_1-TFA derivative.

(f) *Dichlorodimethylsilane (DDS).*—5% in toluene. Add 5 mL DDS (99%) (Aldrich Chemical Co., or equiv.) to toluene and dil. to 100 mL. Store in g-s flask in cold. (*Caution:* DDS is a lachrymator and is flammable.)

26.B04 *Apparatus*

(a) *Silica gel cleanup columns.*—0.8 × 4.0 cm polypropylene Econo-Column with Luer tip, 35 μm, porous polypropylene bed support disk, and 10 mL reservoir (Bio-Rad Laboratories, Cat. No. 731-1550).

(b) *Silica gel cleanup column packing and preparation.*—Dry silica gel 60, particle size 0.040–0.063 mm (E. Merck, No. 9385) in 105° oven for 1 h. Cool and add 1% H_2O by wt. Shake in sealed container and equilibrate overnight before use. Assemble polypropylene column and 250 mL vac. flask fitted with 1-hole stopper as shown in Fig. **26:B1.** Fill column to ca 2 mL mark with silica gel (ca 1 g). Pull gentle vac. to pack bed and add ca 1 g anhyd. Na_2SO_4 to top of silica gel bed.

(c) *Extraction cartridges.*—C18 Sep-Pak sample prepn cartridges (Waters Associates, Inc.).

(d) *Disposable pipet tips.*—50 and 200 μL Eppendorf or equiv.

(e) *Liquid chromatograph.*—Any pulse-free or pulse-dampened liq. chromatgc system which includes pump(s), injector, and compatible recorder.

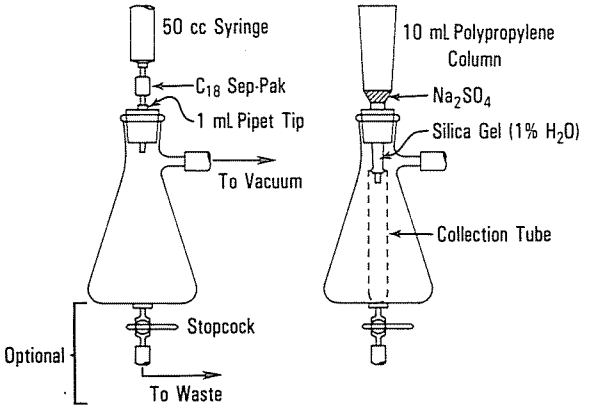

FIG. 26:B1—Diagram of apparatus for extraction and cleanup of milk extracts

(f) *Fluorescence detector.*—Any fluorescence detector capable of providing 365 nm excitation and >400 nm emission wavelengths and sensitivity of 50–100% full-scale response for 1 ng M_1-TFA derivative (e.g., Kratos-Schoeffel FS 970).

(g) *LC analytical column.*—Any 0.4 × 25 cm column contg spherical 5 μm particle size C18 bonded silica gel (e.g., DuPont ODS, Spherisorb 5 ODS II).

(h) *Vacuum regulator.*—Any com. or custom device capable of creating and controlling partial and full vac. with side arm vac. flask.

(i) *Silylated vials for aflatoxin std solns.*—Fill 1 or 1½ dram glass vials nearly full with 5% DDS and heat ca 40 min at 45–55°. Discard soln, and rinse vials 3 times with toluene and then 3 times with MeOH. Heat vials in oven at 75° for 20–30 min to evap. MeOH. Cap vials (with Teflon liners) and store for aflatoxin std solns.

26.B05 *Extraction*

Attach inlet (longer) stem of C18 cartridge to Luer tip of 30–50 mL syringe. Assemble syringe, cartridge, and vac. flask as shown in Fig. **26:B1.** Adjust vac. to pull solvs thru cartridge in fast *dropwise* manner (ca 5 mm Hg). Prime cartridge by adding 5 mL MeOH, then 5 mL H_2O (do not pull cartridge dry; leave small excess H_2O in stem). Discontinue vac. and remove cartridge-syringe assembly from stopper to prevent loss of prime.

Warm sample to room temp. Gently invert sample ≥10 times to evenly distribute cream in nonhomogenized samples. Transfer 20 mL milk to graduate *contg* 20 mL hot (ca 80°) H_2O. (If necessary, more hot H_2O may be used to thin milk soln.)

Replace cartridge-syringe assembly in stopper. Pour entire 40 mL warm, dild sample into syringe and gently pull sample thru cartridge at flow rate ca 30 mL/min (very fast drops). *Caution:* Too fast a flow will not allow sufficient time for aflatoxin to adsorb, resulting in low recoveries. Add 10 mL H_2O–CH_3CN wash soln to syringe and pull thru. Plug syringe barrel with stopper and pull hard vac. on cartridge for ca 30 s to remove as much wash soln as possible from packing. Remove cartridge and dry inside of both stems with cotton swab or tissue paper to eliminate any remaining wash soln. Reprime cartridge by adding 150 μL CH_3CN to inlet bed support disk and let solv. soak into packing for 30 s. Attach cartridge to dry glass or plastic 10 mL Luer tip syringe, retaining same stem as inlet.

Insert silica gel cleanup column into 250 mL vac. flask fitted with 1-hole rubber stopper (Fig. **26:B1**). Wash column with 5 mL ether. Add 7 mL ether to syringe-cartridge positioned above silica gel cleanup column. With plunger, slowly force ether thru cartridge (in portions), collecting eluate in column reservoir. Pull ether slowly thru silica cleanup column, using vac. to maintain flow rate ca 10 mL/min (fast drops). Rinse silica column with 2 mL addnl ether, continuing to use vac. Discard ether.

Remove column and stopper from flask and place 16 × 125 mm collection tube in flask to catch eluate from column. Add 7 mL

Table 3. Collaborative results for determination of aflatoxins M_1 and M_2 (ng/mL) in uncontaminated fluid milk (blind duplicates)[a]

		Sample 4		Sample 11	
Coll.	Method[b]	M_1	M_2	M_1	M_2
1[c]	R	0.10	<0.01	0.02	0
4	R	0	0	0	0
6	R	0	0	0	0
8	N	0	0	0	0
18	R	0	0	0.03	0
19	R	0	0	0	0
20	N	0	0	0.02	0
21	N	0.08	0	tr[d]	0
22	R	0	0	0	0
23	R	0	0	0	0
25	R	0	0	0	0
26	R	tr	0	0	0
27	R	0	0	0	0
33	R	0	0	0	0

	Sample 4		Sample 11		Other samples[e]
Method	M_1	M_2	M_1	M_2	M_2
Total observations:					
	13	13	13	13	78
Positive observations:					
Reverse phase	1	0	1	0	11
Normal phase	1	0	2	0	3
Negative observations:					
Reverse phase	9	10	9	10	49
Normal phase	2	3	1	3	15

[a]Determined by LC method of Foos and Warren (11).
[b]N = normal phase; R = reverse phase.
[c]Values omitted from calculations after applying Youden's ranking test (16).
[d]Collaborator reported trace.
[e]Samples 2/9, 3/10, and 7/14.

elution solv. (CH_2Cl_2–alcohol) to column reservoir. Pull solv. thru column with vac. at ca 10 mL/min flow rate, collecting eluate in tube.

Discontinue vac. and remove collection tube from assembly. Evap. eluate just to dryness under N stream, using heat to keep collection tube near room temp. or under vac. at <35°.

Transfer residue to 1 dram vial with CH_2Cl_2 and evap. to dryness under N on steam bath or in heating block <50°. (Do *not* overheat dry sample.) Save for derivative prepn.

26.B06 *Liquid Chromatography*

Prep. derivative of sample exts by adding 200 μL hexane and 200 μL trifluoroacetic acid to dry residue in vial. Shake on vortex mixer ca 5–10 s. Let mixt. sit 10 min at 40°, in heating block or bath; then evap. to dryness under N on steam bath or heating block (<50°). Add 2 mL H_2O–CH_3CN (75 + 25) to vial to dissolve residue and shake well in vortex mixer for LC analysis. For derivative of std M_1, add 200 μL hexane and 50 μL trifluoroacetic acid to silylated vial and mix. Add 50 μL M_1–M_2 working std soln *directly* into hexane–TFA mixt. and shake on vortex mixer 5–10 s. Treat as described for sample derivative. Stabilize instrument and detector for suitable period at flow rate of 1.0 mL/min with H_2O–isopropanol–CH_3CN (80 + 12 + 8). Adjust detector attenuator so that 50–100 μL injection of std (0.625–1.25 ng M_1, 0.125–0.25 ng M_2) gives 50–75% full-scale recorder pen deflection for aflatoxin M_1. Inject LC std 2–3 times until peak hts are const. Prep. std curve from either peak hts or peak areas to ensure linear relationship. Inject sample exts (typically 50–100 μL) with std injections interspersed to ensure accurate quantitation. Retention times of M_1 (as TFA derivative) and M_2 are ca 4–5 min and ca 7 min, resp.

Calc. aflatoxin concn:

$$\text{ppb } (M_1 \text{ or } M_2) = (H \times C' \times VI' \times V)/(H' \times VI \times W)$$

where H and H' = peak ht of sample and std, resp.; C' = concn of std (ng/μL); VI' and VI = vol. injected of std and sample, resp.; V = final total sample vol. (μL); and W = vol. of milk represented by final ext (typically 20 mL). Sep. calc. concn for M_1 and M_2.

Normal Phase LC Procedure

The normal phase LC procedure, which was not adopted, was performed as follows:

Reagents

(a) *Solvents.*—Distilled-in-glass acetonitrile, chloroform, and methylene chloride; reagent grade diethyl ether (0.01% ethanol preservative), ethanol, and methanol.

(b) *Water–acetonitrile wash solution.*—95 + 5.

(c) *Methylene chloride–ethanol elution solution.*—95 + 5.

(d) *Mobile phase.*—Add 22.5 mL ethanol (reduce by amount in $CHCl_3$ as preservative) to 1 L $CHCl_3$ solution (225 mL water-saturated $CHCl_3$ + 775 mL $CHCl_3$ [ethanol-preserved]). Stir and degas in ultrasonic bath or equivalent.

(e) *Aflatoxin standard solutions.*—Aflatoxin M_1 (Eureka Laboratories, Sacramento, CA 95816) and aflatoxin M_2 (Sigma Chemical Co., St. Louis, MO 63176). Prepare separate stock solutions of each in acetonitrile–benzene (1 + 9) to concentration of 1.0 μg/mL. Store in freezer. Prepare standard daily by diluting aliquots of stock solutions with fresh mobile phase to obtain 1 solution that contains 0.01 μg M_1/mL and 0.004 μg M_2/mL.

(f) *Dichlorodimethylsilane (DDS).*—See **26.B03(f).**

Apparatus

(a) *Silica gel cleanup columns.*—See **26.B04(a).**

(b) *Silica gel cleanup column packing and preparation.*—See **26.B04(b).**

(c) *Extraction cartridges.*—See **26.B04(c).**

(d) *Disposable pipet tips.*—See **26.B04(d).**

(e) *Liquid chromatograph.*—See **26.B04(e).**

(f) *Fluorescence detector.*—Any fluorescence detector with silica gel-packed cell and filters to provide 365 nm excitation and >400 nm emission wavelengths and sensitivity of 50–100% full-scale response for 1 ng M_1 (e.g., Varian Fluorichrom).

(g) *LC analytical column.*—Any 0.4 × 25 cm column containing spherical-shape 5 μm particle size silica gel (e.g., DuPont Zorbax Sil, Varian Micro-Pak SI-5).

(h) *Vacuum regulator.*—See **26.B04(h).**

(i) *Silylated vials for aflatoxin standard solutions.*—See **26.B04(i).**

Extraction

See **26.B05** through next-to-last paragraph. Then dissolve eluate residue in 1000 μL fresh LC mobile phase and mix well.

Liquid Chromatography

Stabilize instrument and detector for suitable period at flow rate of 1.0 mL/min with $CHCl_3$–ethanol mobile phase. Adjust detector attenuator so that 100 μL injection of standard (1.0 ng M_1, 0.4 ng M_2) gives 50–75% full-scale recorder pen deflection for aflatoxin M_1. Inject 100 μL LC standard 2–3 times or until peak heights are constant. Prepare standard curve from either peak heights or peak areas to ensure linear relationship. Inject sample extracts (typically 100 μL) with standard injections interspersed to ensure accurate quantitation. Retention times of M_1 and M_2 are 8–10 min and 9–12 min, respectively, depending on length of column.

Calculate aflatoxin concentration as shown in **26.B06.**

Table 4. Analysis of variance for combined samples[a]

Source of variation	Aflatoxin M_1 df[b]	Aflatoxin M_1 Mean square	Aflatoxin M_2 df	Aflatoxin M_2 Mean square	Expected mean square
Laboratories (L)	9	0.19073***	9	0.26283***	$S_o^2 + 2S_{Ls}^2 + 12S_L^2(M_1)$ or $6S_L^2(M_2)$
Samples (S)	5	4.78924***	2	0.98834***	
L × S	45	0.02105 ns	18	0.03335***	$S_o^2 + 2S_{Ls}^2$
Duplicates	60	0.01145	30	0.00867	S_o^2

Component	Precision Parameters, %[c] M_1	M_2
Repeatability (S_o)	27.9	23.9
Lab-sample interaction (S_{Ls})	17.3	29.1
Among labs (S_L)	31.5	56.9
Reproducibility[d] (S_x)	44.5	64.7

*** = Significant at 0.001 level; ns = not significant

[a]Analysis of variance calculated by log transformation according to Snedecor and Cochran (18). Values are based on logarithms. Normal phase data (Colls. 8, 20, and 21) not included.

[b]df = degrees of freedom

[c]Calculated from antilogarithm formula: $(10^{\sqrt{S_L^2}} - 1) \times 100$; for example, $S_{Ls} = \left(10^{\sqrt{\frac{0.02105 - 0.01145}{2}}} - 1\right) \times 100 = 17.3\%$

[d]$S_x^2 = S_o^2 + S_L^2$

Results and Discussion

Individual values were omitted from calculations according to Dixon's test for outliers at the 0.05 level (15) using either upper or lower 1-tail test (never both on same sample). For statistical calculations based on log transformations, the retained value of the sample pair was substituted for the outlier to maintain balance in the analysis of variance. The "lost" sample (Collaborator 21, sample 5/12) was treated similarly. For aflatoxin M_2 values reported as "trace," a value of 0.005 ng/mL was substituted. The values for Collaborator 1 were not included in the calculations because the composite data exceeded the lower limit of Youden's ranking test (16). The decision to omit these data was made because 11 of the 12 sample values were either the lowest value (7 samples) or the second lowest (4 samples) reported by the collaborators. This was an abnormally high percentage of low values.

The results reported for aflatoxins M_1 and M_2 in artificially contaminated fluid milk are shown in Table 1, and the statistical summaries are given in Table 2. Only 3 collaborators used normal phase LC for their final determination; consequently, separate statistical comparisons between normal and reverse phase LC data were not made. An analysis of variance was done with the reverse phase data only because normal phase data are insufficient to give meaningful results.

The statistical means for the 6 duplicate sets of artificially contaminated samples are shown in Table 2. Aflatoxin M_1 concentrations ranged from 0.072 to 1.235 ng/mL (ppb), while aflatoxin M_2 concentrations varied from 0.038 to 0.128 ng/mL. Levels for both toxins are similar to those reported in contaminated commercial milk. No false negative values were reported for the lowest M_1 concentration (sample 3/10, 0.072 ng/mL); therefore, a minimum detection limit of 70 ppt is realized without modifications of sample extract volumes. Lower detection limits (10–50 ppt) have been obtained (at NRRC) by reducing the final extract volume from 2000 μL to 500–1000 μL.

Aflatoxin M_1 recoveries were excellent (Table 2) and ranged from 85.2 to 102.5%. The average, 93.7%, compares favorably with that of the previous collaborative study (1) (method I, 91% recovery) The standard deviations for M_1 samples are considerably less in this study as are the coefficients of variation (Table 2) (19.2–27.7%, av. 21.4%) compared with those of method I (47%) (1).

Five false positive values, including 2 "trace" values, were reported in this current study for 26 uncontaminated M_1 samples (Table 3). This is comparable to the 2 previous AOAC aflatoxin M_1 collaborative studies (1, 17); however, a lesser number of false positives was expected. Two collaborators (Collaborators 18 and 23) necessarily used contaminated commercial milk and subtracted an "average" background value from their sample concentrations. One reported a false positive (Collaborator 18, sample 4/11, Table 3). Low-level contamination in commercial milk (<0.09 ng M_1/mL) had been determined by several scientists in 1984 (private communications). This problem may have contributed to the larger incidence of normal phase false positives (3 of 6).

Recoveries of aflatoxin M_2 from the artificially contaminated milk were elevated slightly: 99.5, 103.3, and 126.7% (Table 2). The highest recovery (samples 5/12, 126%) was for the lowest level samples, 0.03 ng/mL. Peak heights or areas for aflatoxin M_2 are not large for low concentrations; therefore, a mean of 0.038 ng/mL for a theoretical value of 0.03 ng/mL is satisfactory and acceptable. Aflatoxin M_2 coefficients of variations were twice those for M_1 except for samples 6/13 which were lower (12.5%). This reflects the increased level of M_2 in samples 6/13 (0.128 ng/mL). Since no other study has included aflatoxin M_2, comparisons are not possible. Very little data have been published for aflatoxin M_2 in commercial samples; so, either M_2 is not a contamination problem or scientists are not looking for or reporting it. Of the 76 positive aflatoxin M_2 samples, there were only 6 false negatives (<10%) (Table 1). Four were from the lowest level samples (samples 5/12, 0.03 ng/mL). There were 13 false positives for 78 recorded values (Tables 1 and 3). Eight were found on only one of the possible two in each duplicate set of negative samples. This result indicates that care needs to be exercised when identifying aflatoxin M_2 peaks in milk samples by LC. There were no false positive aflatoxin M_2 values for the uncontaminated milk samples 4/11 (Table 3). Evidently, collaborators realized that study samples without aflatoxin M_1 were not likely to have aflatoxin M_2 either.

The analyses of variance and variance components after combining data for all artificially contaminated samples from reverse phase data are presented in Table 4. A better understanding of the total variance and the component sources of variation is obtained by use of the entire composite of samples. A log transformation was used to compute the analysis

of variance as recommended by Snedecor and Cochran (18) for situations where standard deviations (Table 2) are proportional to means rather than constant. This relation was observed in this study for although the CV values were relatively constant, sample means varied by a factor of about 20. The symbols representing the precision parameters are the ones recommended by the Committee on Collaborative Interlaboratory Studies (19).

The within-laboratory precision parameter, S_o, is the repeatability while the among-laboratory precision parameter, S_x, is the reproducibility. The latter includes both the within-laboratory variance, S_o, and the among-laboratory variance, S_L. For aflatoxin M_1, the repeatability (S_o) was 27.9% which compares favorably with a previous study, 25% (1). The reproducibility, 44.5%, was less than calculated for Method I (47%) (1). It is noteworthy that the lab-sample interaction values are very low for both M_1 and M_2, even though collaborators provided their own fluid milk. The repeatability for aflatoxin M_2 was 23.9% whereas the reproducibility was 64.7%. The latter value is higher than for M_1; however, there is no other M_2 study for comparison.

Generally, the collaborators commented positively about the method. They liked its rapidity and the lack of chromatographic interferences. Only one collaborator (No. 19) did not feel the method was rapid. Four collaborators (Nos. 18, 19, 22, and 25) detected incomplete derivative reactions in the reverse phase LC procedure. Subsequently, these collaborators tried heating the reaction mixture and found no unreacted aflatoxin M_1. Collaborator 22 suggested that the method should incorporate a statement to store the dry film extracts in the freezer until the LC step. Collaborator 25 commented that silylating the vials helped prevent degradation of aflatoxin standards and was in favor of this technique.

Recommendation

The conclusion from the evaluation of this collaborative study indicates that the Foos-Warren method for the rapid determination of aflatoxins M_1 and M_2 in fluid milk by reverse phase LC (11) should be adopted as official first action. The Associate Referee on aflatoxin M recommends that action. Although the data for normal phase LC are satisfactory, they are insufficient to give a meaningful analysis of variance; therefore, normal phase LC cannot be recommended for adoption.

Acknowledgments

The authors thank W. Bailey for statistical processing of the data; M. Sloan, formerly of Waters Associates, Milford, MA, for providing the C_{18} Sep-Pak cartridges; and the following collaborators for their cooperation:

M. Billotte, United Dairymen Association of Arizona, Tempe, AZ

J. Ferguson-Foos and J. D. Warren, Florida Dept of Agriculture and Consumer Services, Tallahassee, FL

J. I. Greer, U.S. Dept of Agriculture, Northern Regional Research Center, Peoria, IL

K. D. Johnson, General Mills, Inc., Minneapolis, MN

J. I. Pitt and N. F. Tobin, Commonwealth Scientific and Industrial Research Organization, North Ryde, Australia

J. L. Richard and R. Lyon, U.S. Dept of Agriculture, National Animal Disease Center, Ames, IA

B. Roberts, Central Veterinary Laboratory, Ministry of Agriculture, Fisheries, and Food, New Haw, Weybridge, Surrey, UK

Y. Saito, National Institute of Hygienic Sciences, Division of Foods, Tokyo, Japan

G. Sutton, South Carolina Dept of Health and Environmental Control, Columbia, SC

M. Trucksess, Food and Drug Administration, Washington, DC

H. P. van Egmond and E. A. Sizoo, National Institute of Public Health, Bilthoven, The Netherlands

N. Webb and M. E. Rhodes, Florida Dept of Agriculture and Consumer Services, Tallahassee, FL

D. Wilson and R. Beaver, University of Georgia, Dept of Plant Pathology, Coastal Plains Experiment Station, Tifton, GA

A. E. Yousef, G. Rusul, and E. H. Marth, Dept of Food Science, University of Wisconsin, Madison, WI

References

(1) Stubblefield, R. D., van Egmond, H. P., Paulsch, W. E., & Schuller, P. L. (1980) *J. Assoc. Off. Anal. Chem.* **63,** 907–921
(2) Stubblefield, R. D. (1979) *J. Am. Oil Chem. Soc.* **56,** 800–802
(3) van Egmond, H. P., Paulsch, W. E., & Schuller, P. L. (1978) *J. Assoc. Off. Anal. Chem.* **61,** 809–812
(4) "Changes in Methods" (1980) *J. Assoc. Off. Anal. Chem.* **63,** 309
(5) Winterlin, W., Hall, G., & Hsieh, D. P. H. (1979) *Anal. Chem.* **51,** 1873–1874
(6) Beebe, R. M., & Takahashi, D. M. (1980). *J. Agric. Food Chem.* **28,** 481–482
(7) Fremy, J. M., & Boursier, B. (1981) *J. Chromatogr.* **219,** 156–161
(8) Tuinstra, L. G. M. T., & Haasnoot, W. (1982) *Fresenius Z. Anal. Chem.* **312,** 622–623
(9) Chang, H. L., & DeVries, J. W. (1983) *J. Assoc. Off. Anal. Chem.* **66,** 913–917
(10) Cohen, H., LaPointe, M., & Fremy, J. M. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 49–51
(11) Ferguson-Foos, J., & Warren, J. D. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 1111–1114
(12) *Official Methods of Analysis* (1980) 13th Ed., AOAC, Arlington, VA
(13) Stubblefield, R. D., Shotwell, O. L., & Shannon, G. M. (1968) *J. Am. Oil Chem. Soc.* **45,** 686–688
(14) Stubblefield, R. D., Shannon, G. M., & Shotwell, O. L. (1971) *J. Am. Oil Chem. Soc.* **47,** 389–390
(15) Dixon, W. J., & Massey, F. J. (1957) *Introduction to Statistical Analysis,* 2nd Ed., McGraw-Hill Book Co., New York, NY, p. 277
(16) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC,* AOAC, Arlington, VA
(17) Stubblefield, R. D., & Shannon, G. M. (1974) *J. Assoc. Off. Anal. Chem.* **57,** 852–857
(18) Snedecor, G. W., & Cochran, W. C. (1980) *Statistical Methods,* 7th Ed., Iowa State University Press, Ames, IA, pp. 291–292
(19) Horwitz, W. (1984) *J. Assoc. Off. Anal. Chem.* **67,** 432

Reprinted from the *Journal of the Association of Official Analytical Chemists.* Vol. 69 #5
Purchased by U.S. Department of Agriculture for official use.